NEW PLANT GENES

FIELD OF THE INVENTION

This invention relates to glutathione transferase (GST) subunits, to nucleic acid sequences encoding glutathione transferase subunits, and to uses of these glutathione transferases and coding sequences, especially in the field of plant biotechnology.

BACKGROUND OF THE INVENTION

Glutathione transferases (GSTs, EC. 2.5.1.18), also referred to as glutathione *S*-transferases, are multifunctional enzymes capable of catalysing the conjugation of electrophilic substrates with the tripeptide glutathione (GSH, gamma-glutamylcysteinylglycine). The electrophilic substrate may be of natural or synthetic origin, examples including endogenous stress-metabolites, drugs, pesticides and pollutants. Conjugation with GSH renders the compounds non-toxic and suitable for export from the cytosol and further metabolism. In addition to their activities in GSH conjugation, GSTs may have additional activities as glutathione peroxidases, catalysing the reduction of organic hydroperoxides to the corresponding alcohol according to the reaction:

R-OOH + 2 GSH -----> R-OH + GSSG.

All known active GSTs are composed of two polypeptide subunits, with each subunit possessing a binding site for GSH and the electrophilic co-substrate. The two subunits may either be identical, giving rise to a homodimer, or dissimilar giving rise to heterodimers. GSTs may therefore be defined according to their source, or class, and their component subunits according to the nomenclature SpGST x-y, where Sp = source or class of GST; x and y describe the subunit types.

Each discrete subunit is encoded by a distinct gene, with many eukaryotes containing

GST multigene families encoding multiple isoenzymes.

The plant in which GSTs have been characterised in the greatest detail is maize (*Zea mays* L.). The major maize GSTs are composed of three discrete subunits, termed I, II and III. These subunits associate together to form three isoenzymes containing the *Zea mays* GST I subunit, namely *Zm*GSTI-I, *Zm*GSTI-II and *Zm*GSTI-III as well as the homodimers *Zm*GSTII-II and *Zm*GSTIII-III. The nucleotide sequences of *Zm*GSTI, *Zm*GSTII and *Zm*GSTIII have been determined. In view of their relatedness in sequence, these maize GSTs have collectively been termed type I plant GSTs. Additional maize GSTs with activities toward herbicides have been described as *Zm*GSTV-V and *Zm*GSTV-VI. The sequence of *Zm*GSTV differs markedly from the other maize GSTs described to date, resembling the auxin-inducible GSTs from dicotyledenous plants which have been termed the type III GSTs.

The maize GST subunit types are associated with differing substrate specificities. The *Zm*GSTI subunit has broad-ranging, but low, activities toward chloro-*s*-triazine, chloroacetanilide and diphenyl ether herbicides. The *Zm*GSTII and *Zm*GSTIII subunits show greater specificity toward chloroacetanilides, while *Zm*GSTV and *Zm*GSTVI are highly active toward diphenyl ethers. The GST isoenzymes differ in their patterns of expression in the organs of maize. Thus, *Zm*GSTI-I and *Zm*GSTV-V are expressed in all plant parts, while *Zm*GSTI-II is root specific. The expression of the GST subunits is also differentially affected by herbicide safeners. These are compounds which enhance the tolerance of cereal crops to herbicides, in part, by increasing the expression of detoxifying enzymes such as GSTs. Thus, the *Zm*GSTII and *Zm*GSTV subunits accumulate in maize seedlings following treatment with the safeners dichlormid or benoxacor while the *Zm*GSTI and *Zm*GSTIII subunits are only modestly enhanced by safeners.

Far less is known regarding GSTs in plant species other than maize. GSTs with activities toward non-herbicide substrates have been identified in some plants, and mRNAs apparently encoding GSTs have been shown to be expressed in plants

including carnation, tobacco and thale cress (*Arabidopsis thaliana*). However, isoenzymes with activities toward herbicides have only been definitively identified in soybean, pea and pine trees. Of these, only in soybean has the nucleotide coding sequences of the herbicide-detoxifying GST been reported.

GSTs in plants have also been shown to have secondary activities as glutathione peroxidases, able to reduce organic hydroperoxides, such as fatty acid hydroperoxides to the corresponding monohydroxy alcohols. GSTs with glutathione peroxidase activity have been isolated from peas, soybean, *A. thaliana* and wheat flour. Since fatty acid hydroperoxides are a common result of membrane peroxidation imposed during oxidative stress, glutathione peroxidases provide an important cytoprotective function in preventing the accumulation of fatty acid hydroperoxides and their subsequent degradation to toxic aldehydes. Glutathione peroxidases may therefore have a vital function in protecting plant cells from oxidative stress. The intervention of glutathione peroxidases in lipid peroxidation has also been cited as a determinant of flour quality in wheat.

Of particular relevance to this invention is the lack of knowledge concerning the GSTs of wheat (*Triticum aestivum* L.).

Some information is available from experiments on whole plants and plant extracts. Several herbicides including examples of the chloroacetanilides, as well as dimethenamid and fenoxaprop-ethyl undergo GSH conjugation in the course of their detoxification in wheat. Also, in crude plant extracts GST activities toward chloroacetanilide herbicides, dimethenamid and fenoxaprop-ethyl have been demonstrated.

There have been very few reports of the purification of GSTs from wheat. A GST was purified from wheat flour, and described as a homodimer of 27.5 kDa polypeptides with activity toward the non-herbicide substrate 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione peroxidase activity toward fatty acid hydroperoxides. A safener-induced

GST with activity toward CDNB and dimethenamid, termed GST*Ta*I-I, has been purified and partially sequenced from the wheat progenitor species *Triticum tauschii*,(Reichers et al, (1997), Plant Physiology, 114, pages 1461 to 1470).

Moreover, very little is known regarding GST genes in wheat. An mRNA originally described as *wir5*, which showed sequence similarity to the type 1 maize GSTs, was identified as accumulating in wheat leaves during the onset of acquired resistance to powdery mildew (*Erysiphe graminis)*. The gene was termed *gstA1* and shown to be similar in genomic organisation to maize *ZmGST1*. The gstA1 polypeptide was expressed in recombinant bacteria and shown to have an apparent molecular mass of 29 kDa. The respective enzyme showed GST activity towards the non-herbicide CDNB, though the activity toward other substrates and activity as a glutathione peroxidase was not reported. An antibody was raised to the recombinant GstA1 and used in Western blotting experiments to show that this GST was specifically induced in wheat leaves by pathogen attack. In contrast, a distinct class of GSTs composed of 25 kDa and 26kDa subunits, which were recognised by an antiserum raised to undefined GSTs in maize, accumulated following exposure to cadmium and the herbicides atrazine, alachlor and paraquat. The activities of these xenobiotic-inducible GSTs in wheat and the corresponding nucleotide sequences were not reported. A cDNA correponding to am mRNA encoding a safener-inducible type III GST has been isolated from Triticum tauschii and had the same amino acid sequence as GSTTaI-I, (Reicher et al, (1997), Plant Physiology, 114, page 1568).

Thus, although wheat is an important crop plant, there has been little molecular characterisation of wheat GSTs or their genes and, to date, only two purified GSTs and two GST gene sequences, *gst*A1 and GSTTa1 available.

Significantly, neither purified recombinant GST proteins expressed from gene *gstA1* or GSTTa1 were reported to exhibit activity towards herbicides. Hence, none of the previous work on wheat GSTs actually provides any means of achieving herbicide resistance based on the function of wheat GSTs.

SUMMARY OF THE INVENTION

We have purified four GST isoenzymes with activity toward herbicides from wheat shoots treated with the herbicide safener fenchlorazole-ethyl and have identified four distinct subunits. In safener-treated shoots, we have found that the predominant GST subunit is a 25 kDa polypeptide, which has been termed *Triticum aestivum* GST 1 (*Ta*GST1). Additionally, two distinct 26 kDa subunits have been identified and termed *Ta*GST2 and *Ta*GST3 and a 24 kDa subunit, termed *Ta*GST4. These subunits associate together to form the active dimeric isoenzymes *Ta*GST1-1, *Ta*GST1-2, *Ta*GST1-3 and *Ta*GST1-4.

In our experiments, the expression of all four isoenzymes was affected by the herbicide safener fenchlorazole-ethyl, although each one responds in a somewhat different way. The *Ta*GST1-1 isoenzyme is the major GST present in the leaves of untreated wheat seedlings, and its expression is increased by approximately 50% following exposure to fenchlorazole-ethyl. *Ta*GST1-4 is expressed at low levels in untreated shoots and its expression is greatly increased by safener application, while *Ta*GST1-2 and *Ta*GST1-3 are only observed following treatment with the safener. All four of these GST isoenzymes have broad-ranging activities toward xenobiotic substrates and all four demonstrate activity towards herbicides and additional activities as glutathione peroxidases able to reduce organic hydroperoxides, with *Ta*GST1-4 being the most active in this respect. Each isoenzyme also has specific properties. Thus, for example, detoxification of one particular herbicide, fenoxaprop-ethyl, is associated with the more strongly safener-inducible *Ta*GST1-2, *Ta*GST1-3 and *Ta*GST1-4 heterodimers, rather than with the *Ta*GST1-1 homodimer.

Furthermore, we have identified, cloned and sequenced cDNAs for the major type III GSTs in wheat, together with cDNAs encoding a range of type I GSTs, all active in herbicide metabolism. This is fundamental to understanding the GST detoxification system in wheat and to exploiting it to generate transgenic herbicide- resistant plants

expressing wheat GSTs. In many previous studies, GST activity could not be linked to specific genes, precluding this approach.

From the sequences of the cDNAs the amino acid sequences of the GST subunits themselves has been deduced.

Accordingly, the invention provides:

a polynucleotide encoding a glutathione transferase (GST) subunit, which polynucleotide comprises a coding sequence capable of hybridising selectively to the coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 to the complement of one of those sequences.

The invention also provides:

a polypeptide which is a GST subunit and comprises the amino acid sequence of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18 or a sequence substantially homologous thereto, or a fragment of either said sequence.

The invention also provides:

a dimeric protein comprising two GST subunits, wherein at least one subunit is a polypeptide of the invention.

The invention also provides:

a chimeric gene comprising a polynucleotide of the invention operably linked to regulatory sequences that allow expression of the coding sequence in a host cell.

The invention also provides:

a vector comprising a polynucleotide of the invention or a chimeric gene of the invention.

The invention also provides:

a cell transformed or transfected with a vector of the invention.

The invention also provides:

a cell having, integrated into its genome, a chimeric gene of the invention.

The invention also provides:

a process for the production of a polypeptide of the invention, which process comprises:

(a) cultivating a cell of the invention under conditions that allow the expression of the polypeptide; and

(b) recovering the expressed polypeptide.

The invention also provides:

a process for the production of a dimeric protein of the invention, which process comprises:

(a) cultivating a cell of the invention under conditions that allow:

(i) the expression of the polypeptide of the invention and, if a further polynucleotide sequence as defined herein is present, optionally the expression of a further GST subunit encoded by a further polynucleotide, and

(ii) the association of the GST subunit polypeptide of the invention with another GST

subunit polypeptide to form a dimeric protein of the invention; and

(b) recovering the dimeric protein so formed.

The invention also provides:

a method of obtaining a transgenic plant cell comprising:

(a) transforming a plant cell with an expression vector of the invention to give a transgenic plant cell,

and optionally,

(a') transforming the cell with one or more further polynucleotide sequences coding for a GST subunit, operably linked to regulatory elements that allow expression of the subunit in the cell.

The invention also provides:

a method of obtaining a first-generation transgenic plant comprising:

(b) regenerating a transgenic plant cell transformed with a vector of the invention to give a transgenic plant.

The invention also provides:

a method of obtaining a transgenic plant seed comprising:

(c) obtaining a transgenic seed from a transgenic plant obtainable by regenerating a transgenic plant cell transformed with a vector of the invention.

The invention also provides:

a method of obtaining a transgenic progeny plant comprising obtaining a second-generation transgenic progeny plant from a first-generation transgenic plant obtainable by regenerating a transgenic plant cell transformed with a vector of the invention, and optionally obtaining transgenic plants of one or more further generations from the second-generation progeny plant thus obtained.

The invention also provides:

a method of obtaining a transgenic progeny plant comprising obtaining a second-generation transgenic progeny plant from a first-generation transgenic plant obtainable by regenerating a transgenic plant cell transformed with a vector of the invention comprising:

(c) obtaining a transgenic seed from a first-generation transgenic plant obtainable by regenerating a transgenic plant cell transformed with a vector of the invention, then obtaining a second-generation transgenic progeny plant from the transgenic seed;

and/or

(d) propagating clonally a first-generation transgenic plant obtainable by regenerating a transgenic plant cell transformed with a vector of the invention to give a second-generation progeny plant;

and/or

(e) crossing a first-generation transgenic plant obtainable by regenerating a transgenic plant cell transformed with a vector of the invention with another plant to give a second-generation progeny plant;

and optionally;

(f) obtaining transgenic progeny plants of one or more further generations from the second-generation progeny plant thus obtained.

The invention also provides:

a transgenic plant cell, first-generation plant, plant seed or progeny plant obtainable by a method of the invention.

The invention also provides:

a transgenic plant or plant seed comprising plant cells of the invention.

The invention also provides:

a transgenic plant cell callus comprising plant cells of the invention, or obtainable from a transgenic plant cell, first-generation plant, plant seed or progeny plant of the invention.

The invention also provides:

use of a polynucleotide of the invention as a selectable marker for detecting transformation of a plant cell.

The invention also provides:

a nucleic acid construct comprising:

(a) a polynucleotide of the invention operably linked to regulatory elements that

allow expression of the coding sequence in a plant cell; and

(b) a site into which a further polynucleotide comprising a coding sequence can be inserted.

The invention also provides:

a vector comprising such a construct.

The invention also provides:

a method of transforming a plant cell or of obtaining a plant cell culture or transgenic plant comprising:

(a) providing an untransformed plant cell which is susceptible to a herbicide whose herbicidal activity is reduced by a dimeric protein of the invention;

(b) transforming the plant cell with a vector comprising:

(i) a polynucleotide of the invention operably linked to regulatory elements that allow expression of the coding sequence in a plant cell; and

(ii) a site into which a further polynucleotide comprising a coding sequence can be inserted;

(c) cultivating the transformed cell under conditions that allow the expression of the polynucleotide (a) in the construct; and/or

(c') regenerating the cell to give a cell culture or plant such that the polynucleotide (a) in the construct is expressed; and

(d) contacting the cell, cell culture or plant with the herbicide whose herbicidal activity is reduced by the dimeric protein of the invention, and to which the untransformed plant cell was susceptible; and

(e) selecting cells, cell cultures or plants that are less susceptible to the herbicide than are corresponding untransformed cells, cell cultures or plants.

The invention also provides:

use of a dimeric protein of the invention in a method of identifying compounds capable of metabolism by a GST.

The invention also provides:

a method of identifying compounds capable of being metabolised by a glutathione transferase comprising:

(a) contacting a candidate compound suspected of being capable of being metabolised by glutathione transferase with glutathione (GSH) in the presence of a dimeric protein of the invention; and

(b) determining whether or not metabolism of the candidate compound takes place.

The invention also provides:

compounds identified by such methods.

The invention also provides:

a kit for detecting compounds capable of being metabolised by a GST

comprising:

(a) reduced glutathione, hydroxymethylglutathione or homoglutathione;

and

(b) a dimeric protein of the invention.

The invention also provides:

an antibody which specifically recognises a polypeptide or dimeric protein of the invention.

The invention also provides:

a nucleic acid probe which selectively hybridises to the sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17.

The invention also provides:

a method of identifying compounds that induce GST expression in graminaceous plants comprising:

(a) contacting a graminaceous plant, or a cell or cell culture thereof, with a candidate compound suspected of being capable of inducing GST expression; and

(b) determining the level of GST expression in the plant, cell or cell culture.

The invention also provides:

compounds identified by such methods.

The invention also provides:

a kit for identifying compounds that induce GST expression in plants by such a method, which kit comprises an antibody of the invention.

The invention also provides:

a method of determining the GST level in a sample of seed or flour comprising:

(i) determining the level of GST protein present by using an antibody of the invention; or

(ii) determining the level of GST mRNA present using a probe of the invention.

The invention also provides:

a method of controlling the growth of weeds at a locus where a transgenic plant of the invention is being cultivated, which method comprises applying to the locus a herbicide whose herbicidal properties are reduced by a dimeric protein of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Anion-exchange chromatography of affinity-purified wheat GSTs.

Chromatography of A: affinity-purified polar GSTs; and B: affinity-purified hydrophobic GSTs on Hi-Trap Q-Sepharose columns eluted with the increasing NaCl gradient shown. The eluent was monitored for A_{280} as shown with the unbroken line and individual fractions assayed for GST activity.

Figure 2. HPLC analysis of wheat GST subunits.

Reversed-phase HPLC analysis of polypeptide subunits present in A, affinity-purified polar GSTs; B, affinity-purified hydrophobic GSTs; C, the isoenzyme *Ta*GST1-1, resolved by anion-exchange chromatography of the affinity-purified polar GSTs.

DETAILED DESCRIPTION OF THE INVENTION

Polynucleotides

The invention provides polynucleotides comprising sequences encoding novel GST subunits, SEQ ID Nos 1, 3, 5, 7, 9, 11, 13, 15 and 17 and sequences that hybridise selectively to these coding sequences thereof or their complementary sequences. It also provides polynucleotide fragments of these sequences that encode polypeptides having GST activity, as defined herein.

A polynucleotide of the invention is capable of hybridising selectively with the coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 or to the sequence complementary to one of those coding sequences. Polynucleotides of the invention include variants of the coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 which can function as GSTs, when dimerised with another GST subunit. Typically, a polynucleotide of the invention is a contiguous sequence of nucleotides which is capable of selectively hybridising to the coding sequence of SEQ ID. No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 or to the complement of that coding sequence.

A polynucleotide of the invention can hybridise to coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 at a level significantly above background. Background hybridisation may occur, for example, because of other cDNAs present in a cDNA library. The signal level generated by the interaction between a polynucleotide of the invention and the coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 is

typically at least 10 fold, preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P. Selective hybridisation is typically achieved using conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C).

A nucleotide sequence capable of selectively hybridising to the DNA coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 or to the sequence complementary to one of those coding sequences will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95%, 98% or 99%, homologous to the coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 or the complement of one of those sequences over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides.

Any combination of the above mentioned degrees of homology and minimum sizes may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher homology over longer lengths) being preferred. Thus for example a polynucleotide which is at least 90% homologous over 25, preferably over 30 nucleotides forms one aspect of the invention, as does a polynucleotide which is at least 95% homologous over 40 nucleotides.

Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or lifespan of polynucleotides of the invention.

Polynucleotides of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will preferably be at least 10, preferably at least 15 or 20, for example at least 25, 30 or 40 nucleotides in length.

Polynucleotides such as a DNA polynucleotide and primers according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques. The polynucleotides are typically provided in isolated and/or purified form.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Genomic clones corresponding to the cDNAs of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 and 17 containing, for example introns and promoter regions are also aspects of the invention and may also be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques, starting with genomic DNA from a wheat (*Triticum aestivum L.)*, cell, e.g. a wheat shoot cell or a cell of a plant of a related *Triticum* species, for example as described by Feldman *et al.*, (Scientific American, (1981), vol. 244(1) pages 98 to 109).

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al*, 1989, Molecular Cloning: a laboratory manual.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of

ways.

Other allelic variants of the wheat sequences of SEQ ID Nos. 1, 3, 5, 7, 9, 11, 13, 15 and 17 including those from *Triticum aestivum L.* species itself related to *Triticum aestivum L.* (cf Feldman *et al, supra*) may be obtained for example by probing genomic DNA libraries made from a range of wheat cells, using probes as described above.

In addition, other plant homologues of SEQ ID Nos. 1, 3, 5, 7, 9, 11, 13, 15 and 17 may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the coding sequence of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 or its complement. Such sequences may be obtained by probing cDNA or genomic libraries from other plant species with probes as described above. Degenerate probes can be prepared by means known in the art to take into account the possibility of degenerate variation between the DNA sequences of SEQ ID Nos. 1, 3, 5, 7, 9, 11, 13, 15 and 17 and the sequences being probed for under conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C).

Allelic variants and species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding likely conserved amino acid sequences. Likely conserved sequences can be predicted from aligning the amino acid sequences of the invention (SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 and 18) with that of other similar GST subunit sequences. The primers will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of SEQ ID Nos. 1, 3, 5, 7, 9, 11, 13, 15 or 17 sequences or allelic variants thereof. This may be useful where, for example, silent codon changes are required to sequences to

optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

The invention further provides double stranded polynucleotides comprising a polynucleotide of the invention and its complement.

Polynucleotides, probes or primers of the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides, probes or primers of the invention and may be detected using techniques known *per se*.

The present invention also provides polynucleotides encoding the polypeptides of the invention described below. Because such polynucleotides will be useful as sequences for recombinant production of polypeptides of the invention, it is not necessary for them to be selectively hybridisable to the coding sequence of sequence SEQ ID Nos. 1, 3, 5, 7, 9, 11, 13, 15 or 17 although this will generally be desirable. Otherwise, such polynucleotides may be labelled, used, and made as described above if desired. Polypeptides of the invention are described below.

Particularly preferred polynucleotides of the invention are those of SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 or 17 and the polynucleotides that are the coding regions within those sequences i.e. the regions which encode the polypeptides of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18.

Polypeptides

A polypeptide of the invention consists essentially of the amino acid sequence set out in SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18 or a substantially homologous sequence, or of a fragment of either of these sequences. In general, the naturally occurring amino

acid sequences shown in SEQ ID Nos. 2, 4, 6, 8, 10, 12, 14, 16 or 18 are preferred. However, the polypeptides of the invention include homologues of the natural sequences, and fragments of the natural sequences and of their homologues, which have GST activity.

The polypeptides of the invention are glutathione transferase (GST) subunits. The invention also provides dimeric proteins comprising two GST subunits wherein at least one subunit is a polypeptide of the invention.

Thus, the polypeptides of the invention are normally functionally active as GSTs when dimerised with another GST subunit. Thus, dimeric proteins of the invention are capable of catalysing the conjugation of the tripeptide glutathione (GSH, gamma-glutamylcysteinyl glycine) and/or related derivatives to an electrophilic substrate of natural or synthetic origin. Related derivatives include homoglutathione (gamma-glutamylcysteinyl alanine) and hydroxymethylglutathione (gamma-glutamylcysteinyl serine).

Optionally, they may also have one or more of the other properties of naturally occurring GSTs including glutathione peroxidase activity as defined above.

Preferably, they have GST activity towards one or more herbicide substrates. For example, they may have activity towards one or more of the following herbicides: Fluorodifen, Fenoxaprop-ethyl, Metolachlor, Alpha-Metolachlor, Acetochlor, Alachlor, Pretilachlor, Fluthiamid, Dimethenamid, *S*-Dimethenamid, Flupyrsulfuron-methyl, Triflusulfuron-methyl, Acifluorfen, Chlorimuron-ethyl, Fomesafen, Atrazine, Simazine, Cyanazine and the sulphatide metabolite of Metribuzin. Particularly preferred herbicides include Fenoxaprop-ethyl, Flupyrsulfuron-methyl, Fluthiamid, Acetochlor, Metolachlor and Alpha-Metolachlor.

Most preferably, a dimeric protein of the invention is able to catalyse the conjugation of GSH to one or more of the following herbicide substrates: Fenoxaprop-ethyl,

Flupyrsulfuron-methyl, fluthiamid, Acetochlor, Metolachlor and Alpha-Metolachlor.

Optionally, a dimeric protein of the invention may be able to catalyse the conjugation of GSH to one or more non-herbicide substrates, for example CDNB. They may also have activity towards phytotoxic non-herbicide substrates.

Optionally, monomeric polypeptides of the invention may have GST activity as defined above, even when not dimerised.

In particular, a polypeptide of the invention may comprise:

(a) the polypeptide sequence of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18;

(b) an allelic variant or species homologue thereof; or

(c) a protein at least 70 80, 90, 95, 98 or 99% homologous to (a) or (b).

An allelic variant will be a variant which will occur naturally in a plant and which will function in a substantially similar manner to the protein of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18, as defined above. Similarly, a species homologue of the protein will be the equivalent protein which occurs naturally in another plant species which can function as GST. Such a homologue may occur in plants other than wheat, particularly monocotyledonous plants such as related *Triticum* species, rice, maize, oats, rye, barley, triticale or sorghum. Within any one species, a homologue may exist as several allelic variants, and these will all be considered homologues of the protein of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18.

Allelic variants and species homologues can be obtained by following the procedures described herein for the production of the polypeptides of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 and 18 and performing such procedures on a suitable cell source e.g. a cell of a wheat genotype carrying an allelic variant, or a cell of a plant of a different another species. It will also be possible to use a probe as defined above nucleotide sequence to probe libraries made from plant cells in order to obtain clones encoding the allelic or

species variants. The clones can be manipulated by conventional techniques to generate a polypeptide of the invention which can then be produced by recombinant or synthetic techniques known *per se*.

A polypeptide of the invention is preferably at least 70% homologous to the protein of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18, more preferably at least 80 or 90% and more preferably still at least 95%, 97% or 99% homologous thereto over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. Methods of measuring protein homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is calculated on the basis of amino acid identity (sometimes referred to as "hard homology").

The sequence of the polypeptides of SEQ ID Nos 2, 4, 6, 8, 10, 12, 14, 16 and 18 and of allelic variants and species homologues can thus be modified to provide polypeptides of the invention.

Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. The modified polypeptide generally retains activity as a GST, as defined herein. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention also include fragments of the above-mentioned full length polypeptides and variants thereof, including fragments of the sequence set out in SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 and 18. Such fragments typically retain activity as a GST.

Other preferred fragments include those which include an epitope. Suitable fragments will be at least about 5, e.g. 10, 12, 15 or 20 amino acids in size. Polypeptide fragments of the polypeptides of SEQ ID Nos. 2, 4, 6, 8, 10, 12, 14, 16 and 18, and allelic and species variants thereof may contain one or more (e.g. 2, 3, 5, or 10) substitutions, deletions or insertions, including conserved substitutions. Epitopes may be determined either by techniques such as peptide scanning techniques already known in the art. These fragments will be useful for obtaining antibodies to polypeptides and dimeric proteins of the invention.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

Polypeptides of the invention may be modified for example by the addition of Histidine residues or a T7 tag to assist their identification or purification or by the addition of a signal sequence to promote their secretion from a cell.

A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g. ^{125}I, ^{35}S, enzymes, antibodies, polynucleotides and linkers such as biotin.

Polypeptides and dimeric proteins of the invention may be chemically modified, e.g. post-translationally modified. For example, they may be glycosylated or comprise modified amino acid residues. Such modified polypeptides and proteins fall within the scope of the terms "polypeptide" and "dimeric protein" of the invention.

Dimeric proteins

The invention also provides dimeric proteins having two GST subunits wherein at least one of the two subunits is a polypeptide of the invention. These dimeric proteins may have two identical subunits of the invention, i.e. they may be homodimeric. Alternatively, they may have two dissimilar subunits; i.e. they may be heterodimeric.

In heterodimers, the two subunits may both be polypeptides of the invention. Alternatively, one subunit may be a polypeptide of the invention, whilst the other is a different GST subunit.

Thus, for example, heterodimeric proteins of the invention may have one subunit which is a polypeptide of the invention, and one which is a known GST subunit from maize (e.g. ZmGSTI, ZmGSTII, ZmGSTIII, ZmGSTIV, ZmGSTV or ZmGSTVI: see above), or another species.

Preferably, the dimeric proteins have two subunits that are polypeptides of the invention. Various combinations of polypeptides of the invention are possible. Preferred combinations include:

*Ta*GST1-1 (SEQ ID No. 2/SEQ ID No. 2);
*Ta*GST1-2 (SEQ ID No. 2/SEQ ID No. 16);
*Ta*GST1-3 (SEQ ID No. 2/SEQ ID No. 18);

being representative of the major combinations found in GSTs in safener-treated

wheat.

The invention also provides dimeric proteins having two subunits as described above which are fusion proteins. In these fusion proteins, the two subunits are joined by a linker polypeptide. Any linker may be used as long as it does not interfere significantly with the correct association of the two subunits or with the GST activity of the dimer. Such fusion proteins will typically be prepared by joining together the polynucleotides encoding the two monomers in the correct reading frame, then expressing the composite polynucleotide coding sequence under the control of regulatory sequences as defined herein. These composite polynucleotide coding sequences are a further aspect of the invention, as are chimeric genes and vectors comprising them, methods of producing them by recombinant means, and cells and plants comprising such vectors or chimeric genes. It will be understood that dimeric proteins of the invention may be such fusion proteins.

Vectors and chimeric genes

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and cultivating the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors. Bacterial cells, especially *E. Coli* are preferred.

Expression vectors

Preferably, a polynucleotide of the invention in a vector is operably linked to regulatory sequences capable of effecting the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. Such expression vectors can be used to

express the polypeptides of the invention.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequences.

Such vectors may be introduced into a suitable host cell to provide for expression of a polypeptide or polypeptide fragment of the invention, as described below.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, preferably a promoter for the expression of the said polynucleotide and optionally an enhancer and/or a regulator of the promoter. For expression in plant cells, one preferred enhancer is the Tobacco etch virus (TEV) enhancer. A terminator sequence may also be present, as may a polyadenylation sequence. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene (e.g. *nptI* or *nptII*) or methotrexate resistance gene for a plant vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo*, for example for generation of transgenic plants of the invention.

So far as plasmid vectors are concerned, plasmids derived from the Ti plasmid of *Agrobacterium tumefaciens* are especially preferred, as are plasmids derived from the Ri plasmid of *Agrobacterium rhizogenes*.

A further embodiment of the invention provides host cells transformed or transfected with the vectors for the replication and expression of polynucleotides of the invention. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (bacterial), plant, yeast, insect or mammalian cells, bacterial and plant cells

being preferred.

Polynucleotides according to the invention may also be inserted into the vectors described above in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense polynucleotides may also be produced by synthetic means. Such antisense polynucleotides may be used in a method of controlling the levels of GSTs having the sequence of SEQ ID No. 2, 4, 6, 8, 10, 12, 14, 16 or 18 or their variants or species homologues *in planta*.

Promoters and other regulatory elements may be selected to be compatible with the host cell for which the expression vector is designed.

Promoters suitable for use in plant cells may be derived, for example, from plants or from bacteria that associate with plants or from plant viruses. thus, promoters from *Agrobacterium spp*. including the nopaline synthase (nos), octopine synthase (ocs) and mannopine synthase (mas) promoters are preferred. Also preferred are plant promoters such as the ribulose bisphosphate small subunit promoter (rubisco ssu), and the phaseolin. promoter. Also preferred are plant viral promoters such as the cauliflower mosaic virus (CAMV) 35S and 19S promoters.

Depending on the pattern of expression desired, promoters may be constitutive or inducible. For example, strong constitutive expression in plants can be obtained with the CAMV 35S or rubisco ssu promoters. Also, tissue-specific or stage-specific promoters may be used to target expression of polypeptides of the invention to particular tissues in a transgenic plant or to particular stages in its development. Chemically inducible promoters such as those activated by herbicide safeners may also be used,for example the maize GST 27 promoter (WO97/11189), the maize In2-1 promoter (WO90/11361), the maize In2-2 promoter (De Veylder *et al*, (1997), Plant Cell Physiology, Vol. 38, pages 568 to 577.

Especially where expression in plant cells is desired, other regulatory signals may also

be incorporated in the vector, for example a terminator and/or polyadenylation site. One preferred terminator is the nos terminator although other terminators functional is the nos terminator in plant cells may also be used.

Additionally, sequences encoding secretory signals or transit peptides may be included. On expression, these elements direct secretion from the cell or target the polypeptide of the invention to a particular location within the cell. For example, sequences may be added to target the expressed polypeptide to the nucleus or plastids (e.g. chloroplasts) of a plant cell.

Chimeric genes

The invention also provides chimeric genes suitable for securing the expression of polypeptides of the invention in a host cell, preferably a plant cell. These comprise a polynucleotide of the invention, operably linked to regulatory sequences that allow its expression in a host cell, preferably a plant cell.

Typically, therefore, a chimeric gene comprises the following elements in 5' to 3' orientation: a promoter functional in a host (preferably plant) cell, as defined above, a polynucleotide of the invention and a terminator functional in said cell, as defined above. Other elements, for example an enhancer, may also be present. These chimeric genes may be incorporated into vectors, as defined above.

Expression in host cells

Expression vectors of the invention may be introduced into host cells using conventional techniques including calcium phosphate precipitation, DEAE-dextran transfection, or electroporation. For plant cells, preferred transformation techniques include electroporation of plant protoplasts, transformation by *Agrobacterium tumefaciens* and particle bombardment. Particle bombardment is particularly preferred for transformation of monocot cells.

Expression in the host cell may be transient although, preferably, integration of the polynucleotide or chimeric gene of the invention into the cell's genome is achieved.

Suitable cells include cells in which the above-mentioned vectors may be expressed. These include microbial cells such as bacteria such as *E. coli*, plant cells, mammalian cells such as CHO cells, COS7 cells or Hela cells, insect cells or yeast such as *Saccharomyces*. Bacterial and plant cells are preferred.

Optionally, cells of the invention may comprise one or more further polynucleotide sequences encoding a GST subunit, operably linked to regulatory sequences, as defined above, that allow expression of the subunit in the cell. Such polynucleotide sequences may be further polynucleotides of the invention or they may encode other GST subunits as defined above with respect to dimeric proteins.

Such polynucleotides may be naturally present in the cell, e.g. if it is a plant cell or they may be introduced artificially, e.g. as defined above.

Such cells allow the production of heterodimeric proteins of the invention where the polynucleotides encode different GST subunits, or the production of monomeric polypeptides of the invention and/or homodimeric proteins of the invention in greater quantities. For example, they may allow the expression of active heterodimeric enzymes.

Cell culture will take place under standard conditions. Commercially available cultural media for cell culture are widely available and can be used in accordance with manufacturers' instructions.

Processes for production of polypeptides and dimeric proteins

The invention provides processes for the production of polypeptides and dimeric

proteins of the invention by recombinant means.

Generally, monomeric GST subunits of the invention spontaneously dimerise to form homodimers and/or heterodimers of the invention. Thus, in general, expression of polypeptides of the invention gives rise to dimers in the first instance. These dimers may be the desired product; alternatively, it may be desirable to separate the monomers. For example, as described below, it may be desired to separate the monomeric subunits of a homodimer in order to combine them with different monomeric subunits, thereby yielding heterodimers.

Processes for the production of polypeptides of the invention may comprise:

(a) cultivating a transformed cell as defined above under conditions that allow the expression of the polypeptide;

and preferably

(b) recovering the expressed polypeptide.

For example, the expressed monomeric peptides may be recovered by denaturation of dimers formed by them, which separates the subunits. Then, the monomers can be recovered and renatured. Typically, they will then redimerise.

Processes for production of dimeric proteins of the invention may comprise:

(a) cultivating a transformed cell as defined above under conditions that allow

(i) the expression of the polypeptide of the invention and, if a further GST subunit-encoding sequence as defined above is present, optionally the expression of a further GST subunit encoded by the further sequence

and preferably

(ii) the association of the GST subunit polypeptide of the invention with another identical GST subunit polypeptide to form a home dimeric protein of the invention; and/or

(ii) the association of the GST subunit polypeptide of the invention with a non-identical GST subunit to form a heterodimeric protein of the invention.

and preferably

(b) recovering the dimeric proteins so formed, and optionally resolving them.

Where only a single type of GST subunit-encoding sequence of the invention is present in the transformed cell, these processes normally give rise to homodimeric proteins of the invention. Where one or more further GST subunit-encoding sequences is present, these processes give rise to heterodimers or to a mixture of some or all of the following: homodimers of each possible type.

Alternatively, dimeric proteins of the invention can be produced by expressing the required polypeptide subunits in separate cells. This typically leads to the production of two different types of homodimer. The desired heterodimer can then be prepared by: mixing the homodimers and denaturing the mixed sample, or by denaturing the homodimers separately and then mixing them; then renaturing the mixed sample. This will typically lead to a mixture of dimeric proteins comprising both possible types of homodimers and also heterodimers comprising one subunit of each type. Similarly, mixtures of greater numbers of types of dimer can be produced in this way if different homodimers are produced in three or more different cells, or if cells that give rise to heterodimers are used.

For these processes, any transformed cell as described above may be used. Bacterial cells are preferred, especially cells of *E. coli*, although other cell types may also be used.

Optionally, the polypeptide or dimeric protein may be isolated and/or purified, by techniques known in the art.

In processes of the invention, any suitable method may be used to denature and/or renature polypeptides of the invention, and suitable methods are well known in the art.

Similarly, where a mixture of polypeptide subunits or dimeric proteins results, these may be resolved or separated by any suitable technique known in the art.

Antibodies

The invention also provides monoclonal or polyclonal antibodies which specifically recognise polypeptides of the invention or dimeric proteins of the invention.

Thus, antibodies of the invention bind specifically to the polypeptides and/or dimers of the invention, preferably to the extent that they distinguish between the polypeptides and/or dimers of the invention and other GST subunits and GSTs.

Monoclonal antibodies may be prepared by conventional hybridoma technology using polypeptides or dimeric proteins of the invention as immunogens. Polyclonal antibodies may also be prepared by conventional means which comprise inoculating a host animal, for example a rat or a rabbit, with a polypeptide of the invention and recovering immune serum. In order that such antibodies may be made, polypeptides may be haptenised to another polypeptide for use as immunogens in animals or humans. For the purposes of this invention, the term "antibody" includes antibody fragments such as Fv, F(ab) and $F(ab)_2$ fragments, as well as single chain antibodies.

Methods of producing transgenic plant cells, plant parts and tissues, plants and seeds of the invention

Transgenic plant cells, plant parts and tissues, plants and seeds of the invention are transgenic in the sense that they have at least one polynucleotide of the invention introduced into them.

The invention provides a method of obtaining a transgenic plant cell comprising transforming a plant cell with an expression vector of the invention to give a transgenic plant cell; and optionally transforming the cell with one or more further polynucleotide sequences coding for a GST subunit, operably linked to regulatory elements that allow expression of the subunit in the cell.(As discussed above, this allows the production of heterodimeric GST dimers of the invention, or the production of homodimeric ones of the invention in greater quantities.)

Any suitable transformation method may be used, for example the transformation techniques described herein. Preferred transformation techniques include electroporation of plant protoplasts, transformation by *Agrobacterium tumefaciens* and particle bombardment. Particle bombardment is particularly preferred for transformation of monocot cells.

The cell may be in any form. for example, it may be an isolated cell, e.g. a protoplast, or it may be part of a plant tissue, e.g. a callus, or a tissue excised from a plant, or it may be part of a whole plant. Transformation may thus give rise to a chimeric tissue or plant in which some cells are transgenic and some are not.

Preferably, integration of a polynucleotide or chimeric gene of the invention into the cell's genome is achieved.

The thus obtained cell may be regenerated into a transgenic plant by techniques known in the art. These may involve the use of plant growth substances such as auxins,

giberellins and/or cytokinins to stimulate the growth and/or division of the transgenic cell. Similarly, techniques such as somatic embryogenesis and meristem culture may be used.

In many such techniques, one step is the formation of a callus, i.e. a plant tissue comprising expanding and/or dividing cells. Such calli are a further aspect of the invention as are other types of plant cell cultures and plant parts. Thus, for example, the invention provides transgenic plant tissues and parts, including embryos, meristems, seeds, shoots, roots, stems, leaves and flower parts. These may be chimeric in the sense that some of their cells are transgenic and some are not.

Regeneration procedures will typically involve the selection of transformed cells by means of marker genes. Some marker genes have already been mentioned and it should also be noted that the polynucleotides of the invention can themselves act as marker genes if they are under the control of regulatory sequences that allow their expression during the appropriate stage of the regeneration procedure. The polypeptides of the invention are capable of conferring resistance to herbicides or other phytotoxic compounds which are detoxified by GSTs on cells of the invention, as described below. Thus, an appropriate herbicide can be used to select transformants.

The regeneration step gives rise to a first generation transgenic plant. The invention also provides methods of obtaining transgenic plants of further generations this first generation plant. These are known as progeny transgenic plants. progeny plants of second, third fourth, fifth, sixth and further generations may be obtained from the first generation transgenic plant by any means known in the art.

Thus, the invention provides a method of obtaining a transgenic progeny plant comprising obtaining a second-generation transgenic progeny plant from a first-generation transgenic plant of the invention, and optionally obtaining transgenic plants of one or more further generations from the second-generation progeny plant thus obtained.

Such progeny plants are desirable because the first generation plant may not have all the characteristics required for cultivation. For example, for the production of first generation transgenic plants, a plant of a taxon that is easy to transform and regenerate may be chosen. It may therefore be necessary to introduce further characteristics in one or more subsequent generations of progeny plants before a transgenic plant more suitable for cultivation is produced.

Progeny plants may be produced form their predecessors of earlier generations by any known technique. In particular, progeny plants may be produced by:

obtaining a transgenic seed from a transgenic plant of the invention belonging to a previous generation, then obtaining a transgenic progeny plant of the invention belonging to a new generation by growing up the transgenic seed;

and/or

propagating clonally a transgenic plant of the invention belonging to a previous generation to give a transgenic progeny plant of the invention belonging to a new generation;

and/or

crossing a first-generation a transgenic plant of the invention belonging to a previous generation with another compatible plant to give a transgenic progeny plant of the invention belonging to a new generation;

and optionally;

obtaining transgenic progeny plants of one or more further generations from the

progeny plant thus obtained.

These techniques may be used in any combination. for example, clonal propagation and sexual propagation may be used at different points in a process that gives rise to a transgenic plant suitable for cultivation. In particular, repetitive back-crossing with a plant taxon with agronomically desirable characteristics may be undertaken. Further steps of removing cells from a plant and regenerating new plants therefrom may also be carried out.

Also, further desirable characteristics may be introduced by transforming the cells, plant tissues, plants or seeds, at any suitable stage in the above process, to introduce desirable coding sequences other than the polynucleotides of the invention. this may be carried out by the techniques described herein for the introduction of polynucleotides of the invention.

For example, further transgenes may be selected from those coding for other herbicide resistance traits; e.g. tolerance to Glyphosate (e.g. using an EPSP synthase gene (e.g. EP-A-0 293,358) or a glyphosate oxidoreductase (WO 92/000377) gene); or tolerance to fosametin; a dihalobenzonitrile; glufosinate (e.g. using a phosphinotricyine acetyl transferase or glutamine synthase gene (cf. EP-A-0 242,236); asulam (e.g. using a dihydropteroate synthase gene (EP-A-0 369,367); or a sulphonylurea (e.g. using an ALS gene); diphenyl ethers such as acifluorfen or oxyfluorfen (e.g. using a protoporphyrogen oxidase gene); an oxadiazole such as oxadiazon; a cyclic imide such as chlorophthalim; a phenyl pyrrazole such as TNP, or a phenopylate or carbamate analogue thereof.

Similarly, genes for beneficial properties other than herbicide tolerance may be introduced. For example, genes for insect resistance may be introduced, notably genes encoding *Bacillus thuringiensis* (*Bt*) toxins.

Transgenic plant cells, plant parts and tissues, plants and seeds of the invention

The invention also provides transgenic plant cells, plant parts and tissues, plants and seeds. these are typically obtainable, or obtained, by the methods described above. They may be of any botanical taxon, e.g. any species or lower taxonomic grouping. Preferably, they are of a crop pant species.

Transgenic plant cells, plant parts and tissues, plants and seeds of the invention may thus be of a monocotyledonous (monocot) or dicotyledonous (dicot) taxon. Preferred dicot crop plants include tomato; potato; sugarbeet; cruciferous crops, including oilseed rape; linseed; tobacco; sunflower; fibre crops such as cotton; and leguminous crops such as peas, beans, especially soybean, and alfalfa. Preferred monocots include graminaceous plants such as wheat, maize, rice, oats, barley and rye, sorghum, triticale and sugar cane. Wheat is particularly preferred.

Typically, a polypeptide of the invention is expressed in a plant of the invention. depending on the promoter used, this expression may be constitutive or inducible, e.g. by a herbicide safener. similarly, it may be tissue- or stage-specific, i.e. directed towards a particular plant tissue or stage in plant development.

Preferably, plant cells, plant parts and tissues, plants and seeds of the invention exhibit herbicide resistance due, at least in part, to expression of a polypeptide of the invention.

Herbicides to which plants of the invention may be resistant include Fluorodifen, Fenoxaprop-ethyl, Metolachlor, Alpha-Metolachlor, Acetochlor, Alachlor, Pretilachlor, Fluthiamid, Dimethenamid, *S*-Dimethenamid, Flupyrsulfuron-methyl, Triflusulfuron-methyl, Acifluorfen, Chlorimuron-ethyl, Fomesafen, Atrazine, Simazine, Cyanazine, and Metribuzin. Particularly preferred herbicides include Fenoxaprop-ethyl, Flupyrsulfuron-methyl, Fluthiamid, Acetochlor, Metolachlor and Alpha-Metolachlor. Plants of the invention may also exhibit resistance to other herbicides capable of

conjugation to GSH by GSTs or to other non-herbicide phytotoxic substances.

Preferably, a transgenic plant of the invention exhibits resistance to one or more of Fenoxaprop-ethyl, Flupyrsulfuron-methyl, Fluthiamid, Acetochlor, Metalochlor and Alpha-Metolachlor. Resistance may be exhibited to herbicides which are selective for particular plant taxa and/or herbicides which are generic to all plants.

Uses of the polynucleotides, polypeptides, antibodies, probes and plants of the invention

Apart from enabling the generation of herbicide- resistant plants, the invention has a number of other uses.

Selectable markers

Polynucleotides of the invention can be used as selectable markers for detecting the transformation of plant cells. When expressed from polynucleotides of the invention, the polypeptides of the invention are capable of conferring herbicide resistance on cells of the invention, as described herein. Thus, an appropriate herbicide can be used to select transformants.

Accordingly, the invention provides a nucleic acid construct comprising:

(a) a polynucleotide of the invention operably linked to regulatory elements that allow expression of a polynucleotide of the invention a plant cell; and

(b) a site into which a further polynucleotide comprising a coding sequence can be inserted.

Preferably, site (b) is bounded by regulatory elements that allow expression of a coding sequence inserted at the site in a plant cell.

These constructs may be contained within vectors as described herein.

In these constructs, site (b) is a site into which another nucleic acid sequence can be inserted. in cells transformed with the constructs or vectors containing them, expression of the polypeptide of the invention can be used as a selectable marker, indicating that the polynucleotide at site (b) has also been successfully introduced.

In this connection, the invention also provides a method of transforming a plant cell or of obtaining a plant cell culture or transgenic plant comprising:

(a) providing an untransformed plant cell which is susceptible to a herbicide whose herbicidal activity is reduced by a dimeric protein of the invention;

(b) transforming the plant cell with a vector comprising a marker construct of the invention;

(c) cultivating the transformed cell under conditions that allow the expression of a polypeptide of the invention;

and /or

(c') regenerating the cell to give a cell culture or plant such that a polypeptide of the invention is expressed;

and

(d) contacting the cell, cell culture or plant with the herbicide whose herbicidal activity is reduced by a dimeric protein of the invention, and to which herbicide the untransformed plant cell was susceptible;

and

(e)selecting cells, cell cultures or plants that are less susceptible to the herbicide than are corresponding untransformed cells, cell cultures or plants.

Identification of novel herbicides

The polypeptides and dimeric proteins of the invention may be used to identify compounds capable of conjugation to GSH. Thus, as conjugation to GSH is the mechanism by which GSTs are believed to effect detoxification of herbicides, the polypeptides of the invention can be used to determine whether or not a candidate herbicidal compound will be detoxified by GSTs, for example the dimeric proteins of the invention. In this case, it may be possible to develop the candidate compound as a herbicide. In particular, it may be possible to develop the candidate compound for selective use as a herbicide on crops of wheat, or of a wheat-related species, or of other plants (cf Feldman *et al supra*), having GSTs with similar activity to the dimeric proteins of the invention. This is because species having such GSTs can be expected to detoxify herbicides identified in the assay.

Accordingly, the invention provides a method of identifying compounds capable of conjugation to glutathione comprising:

(a) contacting a candidate compound suspected of being capable of being metabolised by glutathione transferase with glutathione (GSH) in the presence of a dimeric protein of the invention; and

(b) determining whether or not metabolism of the candidate compound takes place, or to what extent takes place.

Preferably, metabolism of the compound is detected by determining whether, or to what extent, conjugation of the candidate compound to GSH takes place.

Such assay methods may be carried out by any suitable means known in the art. Compounds may be assayed singly, or, preferably, in batches containing several compounds. For example, microtitre plate-based assay techniques may be used. More specifically, the techniques of Example 4 below may be used.

The invention also provides compounds identified by the methods of the invention.

The invention also provides a kit for detecting compounds capable of being metabolised by a GST comprising:

(a) reduced glutathione, hydroxymethylglutathione or homoglutathione; and

(b) a dimeric protein of the invention.

Such kits may also comprise other components, especially buffer solutions, e.g. aqueous solutions buffered at a suitable pH (e.g. pH7 to pH10, preferably pH7 to pH8).

These kits can be used in the identification of novel herbicides.

Identification of compounds that induce GST expression

We have found that expression of the GSTs of the invention is induced by herbicide safeners. As GSTs are implicated in herbicide resistance, it may be desirable to identify other compounds capable of inducing their expression or that of related GSTs in wheat or other plants, preferably graminaceous plants. Such compounds may, for example, be used to induce expression of GSTs involved in herbicide tolerance. This will be beneficial as it will allow crop plants to be selectively protected from herbicides whilst weeds are killed by them.

Accordingly, the invention provides a method of identifying compounds that induce

GST expression in graminaceous plants comprising:

(a) contacting a plant, preferably a graminaceous plant, or a cell or cell culture thereof, with a candidate compound suspected of being capable of inducing GST expression; and

(b) determining the level of GST expression in the plant, cell or cell culture.

Typically, the level of expression is also determined before the compound is added, or in an untreated sample, in order to provide a control. If the level of GST expression in the test sample is higher than that in the control sample then the candidate compound is an inducer of GST expression.

Preferably, the level of GST expression is determined quantitatively although, in certain situations, quantitative detection may suffice, e.g. where the level of expression is zero or undetectable in the absence of an inducer.

Determination of the level of GST expression may be performed by any suitable means. Preferably, it is performed using antibodies or probes of the invention, as described herein.

The invention also provides compounds identified by these methods.

Antibodies that specifically recognise the polypeptides or dimeric proteins of the invention can be used to detect and preferably quantify GST expression by detecting them directly. The antibodies of the invention may thus be used for detecting polypeptides or dimeric proteins of the invention present in plant samples, e.g. by a method which comprises:

(a) providing an antibody of the invention;

(b) incubating a plant sample with said antibody under conditions which allow for

the formation of an antibody-antigen complex; and

(c) determining, by any suitable technique known in the art, whether antibody-antigen complex comprising said antibody is formed.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

Similarly, polynucleotides or primers of the invention or fragments thereof, labelled or unlabelled, may be used by a person skilled in the art in nucleic acid-based tests for detecting nucleic acid sequences of the invention in a sample taken from a plant, typically a wheat plant.

Such tests generally comprise bringing a sample containing DNA or RNA into contact with a probe comprising a polynucleotide or primer of the invention under hybridising conditions and detecting any duplex formed between the probe and nucleic acid in the sample. Such detection may be achieved using techniques such as PCR or by immobilising the probe on a solid support, removing nucleic acid in the sample which is not hybridised to the probe, and then detecting nucleic acid which has hybridised to the probe.

Alternatively, the sample nucleic acid may be immobilised on a solid support, and the amount of probe bound to such a support can be detected.

The probes of the invention may conveniently be packaged in the form of a test kit in a suitable container. In such kits the probe may be bound to a solid support where the assay format for which the kit is designed requires such binding. The kit may also contain suitable reagents for treating the sample to be probed, hybridising the probe to nucleic acid in the sample, control reagents, instructions, and the like.

Measuring the level of GST in batches of seed or flour

Owing to the secondary activity of the GSTs of the invention as glutathione peroxidases, the polypeptides and dimeric proteins of the invention will also have applications in determining the quality of batches of seed and flour, especially of wheat seed, grain and wheat flour. In such batches, glutathione peroxidases are involved in reducing lipid hydroperoxides, which reduces the amount of GSH available. In particular, this occurs during bread making. Thus, it is desirable to be able to monitor the level of GSTs having glutathione peroxidase activity in batches of seed and flour.

This can be done by any suitable means. For example, antibodies of the invention can be used to detect polypeptides or dimeric proteins of the invention, as described above. Similarly, probes of the invention can be used to detect GST mRNA, as described above.

Alternatively, to determine directly the level of GSH in a batch, the invention provides a method of determining the GSH level in a batch of seed or flour comprising:

(a) contacting a sample from the batch with a polypeptide or dimeric protein of the invention and a compound whose conjugation to GSH is catalysed by the polypeptide or protein; and

(b) determining the GSH level from the extent of reaction between the compound and GSH.

Controlling the growth of weeds

The invention also provides a method of controlling the growth of weeds at a locus where a transgenic plant of the invention is being cultivated, which method comprises applying a herbicide to the locus. Any amount of herbicide may be used, as long as it is herbicidally effective against the weeds but leaves the herbicide resistant plants of the

invention unaffected, or substantially unaffected. The effect on the weeds may be, for example, to kill them or to inhibit their growth.

Any type of weed that responds to a particular herbicide may be controlled in this way. *Alopecurus myosuroides, Avena fatua, Lolium spp., Bromus spp., Poa annua, Galium aparine, Aper spica-venti, Matricaria inodora, Stellaria media, Papaver rhoeas, Polygonum spp., Setaria sp., Sorghum halapense, Panicum miliaceum, Echinochloa spp., Digitaria sanguinalis, Phalaris minor, Abutilon theophrasti, Amaranthus retroflexus, chenopodium album, Datura stramoniuon, Solanum nigrum, Xanthium strumarium, saggitaria spp., Monochoria vaginalis, Lindernia spp., Eleokaris kurogaai, Scirpus juncoides, Cyperus spp.*

The herbicide may, for example, be one whose activity is identified by the methods of the invention (see above). Alternatively, it may be a known herbicide, for example one of the herbicides mentioned herein.

The herbicide may be applied at any suitable time during the life cycle of the transgenic plant, for example pre-emergence or post-emergence. Timing of application will be tailored to the development of the weeds which it is desired to control. Where inducible or tissue- and/or stage- specific expression of the active dimer of the invention is employed, timing of herbicide application will be tailored to the optimal expression of the invention in the course of the development of the transgenic plant of the invention.

The following Examples illustrate the invention.

EXAMPLES

Example 1: Isolation and characterisation of the nucleotide sequence 1 encoding *Ta*GST1

(a) Purification of wheat GST isoenzymes

Wheat GST isoenzymes containing the *Ta*GST1 subunit were purified by the method of Dixon *et al* (Pestic. Sci. 1997, 50, 72-82). This is summarised below.

Wheat seeds (*Triticum aestivum* L. var. Hunter) were imbibed in a 10 mg/l solution of the herbicide safener fenchlorazole-ethyl and then grown in an environmental growth room with further root-applied watering treatments of 5 mg/l fenchlorazole ethyl applied as required. At 10 days after imbibing, the shoot tissue was harvested and extracted prior to precipitation of the protein with ammonium sulphate (80% saturation). The total protein extract was then applied in the presence of 1 M ammonium sulphate to a phenyl-Sepharose column. The bound GSTs were then recovered, firstly by reducing the ammonium sulphate concentration to 0 M to yield the polar GST fraction, which represented 61% of the recovered activity toward 1-chloro-2,4-dinitrobenzene (CDNB). The remaining 39% of the GST activity was then recovered by adding ethylene glycol (50 % v/v) to the running buffer to yield the hydrophobic GST fraction.

The polar and hydrophobic GST fractions were then independently applied to the affinity matrix, *S*-hexyl-glutathione agarose. This matrix bound 90% of the GST activity toward CDNB. Prior to elution of the column with the ligand, *S*-hexyl-glutathione, the matrix was washed with phosphate buffer, followed by phosphate buffer containing 200 mM potassium chloride. The GSTs eluting in this salt wash were termed the "loosely-bound" fraction. Tightly-bound proteins were then eluted with 5 mM *S*-hexyl-glutathione. With both the polar and hydrophobic GSTs an average of 34% of the GST activity toward CDNB eluted in the loosely-bound fraction and 66% eluted in the presence of *S*-hexyl-glutathione. The loosely-bound fraction contained the

GSTs which will be considered in Example 2. The major wheat GSTs of interest in this example were found in the affinity-purified pool and to define the numbers of isoenzymes and component subunits present, this pool was analysed in detail.

When the affinity-bound pools of the polar and hydrophobic GSTs were analysed by anion-exchange chromatography on Q-sepharose, the partial resolution of the eluting activity suggested the presence of multiple isoenzymes (Figure 1). The component polypeptides in the active fractions were then analysed by silver staining after resolution by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). It was concluded that in fenchlorazole-ethyl-treated wheat the polar GSTs were composed of 25 kDa and 26 kDa polypeptides, while the hydrophobic fraction contained 25kDa, 26 kDa and 24 kDa polypeptides. Further analysis by reversed-phase HPLC confirmed the subunit compositions (Figure 2). Based on the combined analyses by Q-sepharose, HPLC and SDS-PAGE these GST polypeptides were named as described in Table 1, which also contains details of how these subunits associate together to form the active dimers found in plants and the relative abundance of these subunits in extracts from unsafened and fenchlorazole-ethyl treated (safened) plants.

(b) GST activities of the purified *Ta*GST isoenzymes

The purified isoenzymes were assayed for GST activity toward herbicides using the HPLC-based assays described by Edwards R. and Cole D.J. (Pesticide Biochemistry and Physiology Vol. 54, pp96-104 (1996)) and the results are presented in Table 2. Both polar and hydrophobic GSTs from the affinity-bound pools of isoenzymes showed detoxifying activities toward the selective graminicide fenoxaprop-ethyl, the diphenyl-ether herbicide fluorodifen, and the chloracetanilide metolachlor. These isoenzymes had additional activities as glutathione peroxidases able to reduce linoleic acid hydroperoxide, a major reaction product formed during membrane peroxidation in plants (Williamson and Beverly. J. Cereal Sci. 8, 1988, 155-163).

(c) Preparation of polyclonal antibodies to the major wheat GST isoenzymes

Purified *Ta*GST1-1 was used to immunise rabbits to raise polyclonal antibodies to the differing isoenzymes. The reactivity of the antiserum toward crude wheat preparations was demonstrated with a Western blot of polypeptides resolved by SDS-PAGE. The antibodies were then used to identify the corresponding cDNAs in an expression library.

(d) Identification and characterisation of a cDNA encoding *Ta*GST1

An expression library was prepared from poly (A)+ RNA extracted from 7-day wheat shoots grown from seed treated with fenchlorazole-ethyl. The library was constructed in lambda ZAP II (Stratagene) and plaque forming units (pfus) screened with the antiserum raised against *Ta*GST1-1. From an initial screen of 170,000 pfus 17 positive plaques were identified, of which 12 were further purified to homogeneity in secondary and tertiary screens and the wheat cDNAs excised from the phage to form Bluescript plasmids in *E. coli* SOLR. (Stratagene). Automated DNA sequencing showed that all clones had an identical coding sequence, although differences in the 5' and 3' untranslated regions were apparent, such that of 6 clones sequenced fully on both strands, 4 different untranslated regions were observed. Since these clones shared a common open reading frame they were all designated *TaGST1* and then subdivided as A, B, C and D. The nucleotide sequence of *TaGST1* showing the variable untranslated regions of A, B, C and D is shown in SEQ ID No. 1, together with the deduced amino acid sequence of the coding region (SEQ ID No. 2).

To confirm that *TaGST1* encoded a GST, it was expressed as a fusion protein with beta-galactosidase using the pBluescript plasmid in *E. coli* strain SOLR. *TaGST1* clones were inoculated into LB liquid medium and were grown overnight at 37/C on an orbital shaker in the presence of IPTG. Bacteria were then pelleted by centrifugation, lysed by sonication and assayed for GST activity toward CDNB and analysed by SDS-PAGE and Western blotting using the anti-*Ta*GST1-1 serum. With all six *TaGST1* clones, GST activity toward CDNB could be determined in the crude extracts in the

range 30 - 50 nkat/ mg crude lysate. This was in contrast to control *E. coli* containing the bluescript plasmid without a cDNA insert which showed negligible GST activity (0.2 nkat/mg). When the polypeptides contained in the lysates of the various *Ta*GST1 clones were analysed by SDS-PAGE, in every case the *Ta*GST1-fusion protein was clearly visible as a highly expressed polypeptide relative to the controls. All the fusion proteins reacted with the anti-*Ta*GST1 serum.

To confirm that the GST activity in the extracts from *Ta*GST1 clones was due to the fusion protein, the GST-fusion was purified using *S*-hexyl-glutathione agarose affinity chromatography. The pure fusion protein was then assayed for enzyme activity toward herbicide and hydroperoxide substrates and was found to show a similar spectrum of activities to that of the pure *Ta*GST1-1 isoenzyme from wheat shoots.

Table 1

Summary of the characteristics of major classes of wheat GST isoenzymes.

The GST subunits had the following retention times by reversed-phase HPLC. *Ta*GST1a - 26.4 min, *Ta*GST1b - 27.1 min, *Ta*GST2 - 31.1 min, *Ta*GST3 - 30.9 min, *Ta*GST4 - 33.2 min.

ISOENZYME TYPE	SUBUNITS	POLAR (P) OR HYDROPHOBIC (H)	MOLECULAR WEIGHT (KDA)	ANTI-TAGST1 ANTIBODY REACTION	% ENHANCEMENT BY SAFENER
TaGST1-1	TaGST1a	P	25	+	30-50
	TaGST1b	P	25	+	30-50
TaGST1-2	TaGST1a	P	25	+	Only
	TaGST1b	P	25	+	observed
	TaGST2	P	26	-	with
					safener
TaGST1-3	TaGST1a	P	25	+	Only
	TaGST1b	P	25	+	observed
	TaGST3	H	26	-	with
					safener
TaGST1-4	TaGST1a	P	25	+	300%
	TaGST1b	P	25	+	300%
	TaGST4	H	24	-	300%

Table 2

Activity of GST isoenzymes purified from fenchlorazole-ethyl-treated wheat shoots.

Enzyme activities are expressed as $nkat.mg^{-1}$

ISOENZYME	CDNB	FLUORODIFEN	FENOXAPROP -ETHYL	METOLACHLOR
Polar				
TaGST1-1	1,528	0.97	0	0.11
TaGST1-2	1,441	0.38	0.61	0.25
Hydrophobic				
TaGST1-3	1,700	0.38	0.44	0.28
TaGST1-4	1,553	0.57	0.23	0.23

Table 3

CDNB and herbicide activities of recombinant wheat GSTs

Activities expressed as nkat.mg^{-1} ± standard error

RECOMBINANT ENZYME	CDNB	FLUORODIFEN	FENOXAPROP-ETHYL	METOLACHLOR
TaGST1	1970 ± 30	2.0 ± 0.1	0	0.127 ± 0.014
WIC 1	406.5 ± 0.5	0.136 ± 0.011	0.050 ± 0.010	0.315 ± 0.003
WIC 2	187 ± 1	0.096 ± 0.002	0.085 ± 0.002	0.512 ± 0.04
WIC 3	2,519 ± 88	0.014 ± 0.006	0.093 ± 0.002	0.053 ± 0.004
WIC 4	980 ± 86	0.036 ± 0.004	0.012 ± 0.001	0.037 ± 0.003
WIC 5	174 ± 8	0.030 ± 0.002	0.067 ± 0.003	0.040 ± 0.004
TA 27	237 ± 13	0.034 ± 0.003	0.036 ± 0.004	0.063 ± 0.006
ICR	8139 ± 146	0.037 ± 0.002	0.028 ± 0.001	0.000 ± 0.000
ICC/V/P	30 ± 4	0.000 ± 0.000	0.074 ± 0.008	0.000 ± 0.000

EXAMPLE 2: Cloning of wheat GSTs resembling the type I GSTs from maize.

(a) Characterisation of type I GSTs in wheat

The observation that extracts from safener-treated wheat shoots contained GSTs which, unlike those described in Example 1, were not selectively retained on the affinity matrix suggested that a discrete class of GSTs were present in this loosely bound fraction. Crude extracts of wheat seedlings were analysed by Western blotting following SDS-PAGE using a polyclonal rabbit antiserum raised to the type I *Zm*GSTI-II heterodimer. The antiserum reacted strongly with several polypeptides of Mr 23 - 27 kDa. These polypeptides were present in the loosely-bound fraction from the *S*-hexyl-glutathione affinity column, but not in the affinity bound fraction.

(b) Cloning of cDNAs from a wheat expression library

Having established that safener-treated wheat shoots contained polypeptides which cross-reacted with the antiserum raised to the maize GSTs, the primary cDNA expression library prepared from fenchlorazole-ethyl treated wheat shoots was screened with the antibody for positive clones. Following a screen of 170,000 pfu., ten positive plaques were identified, with obvious differences in the intensity of recognition, with four plaques showing a strong colour reaction and six plaques of lower intensity. These cDNA clones were termed *WIC* clones. All four of the stronger-reacting plaques (*WIC* 1, 2, 4 and 5) and four of the weaker positives (WIC 3, 7, 8 and 10) were purified to homogeneity, the respective plasmids excised and DNA preparations sequenced. The clones were then grouped by their degree of similarity in sequence.

In the sequence listing, WIC 1 is SEQ ID No. 3 and its deduced amino acid sequence is SEQ ID No. 4. WIC 2 is SEQ ID No. 5 and its deduced amino acid sequence is SEQ ID No. 6. The coding sequences of WIC 3, WIC 7 and WIC 8 were identical in sequence. The DNA sequence of WIC 3/7/8 is given in SEQ ID No. 7 and the deduced amino acid sequence in SEQ ID No. 8 All three sequences contained a stop codon in

the 5' untranslated region of the GST gene, although some expression occurred. The DNA sequence of WIC 5 is shown in SEQ ID No. 9, and the deduced amino acid sequence in SEQ ID No. 10. WIC 4 and WIC 10 had identical coding sequences, but differed in their untranslated regions. In particular, WIC 10 had a stop codon in the 5' untranslated region, though this did not prevent all expression of the fusion protein The WIC 4 DNA sequence is given in SEQ ID No. 11 and the deduced WIC 4/10 amino acid sequence in SEQ ID No. 12 (the WIC 10 DNA sequence is not shown).

(c) Cloning of wheat GSTs by differential screening of a cDNA library

A further cDNA clone, termed *TA 27* was obtained. A cDNA library prepared from wheat seedlings treated with the herbicide safener cloquintocet-mexyl, was screened for clones which represented mRNAs which were differentially expressed in wheat in response to safener application. The identity of the clone as a GST was suggested from its nucleotide (SEQ ID No. 13) and deduced amino acid (SEQ ID No. 14) sequence. As the coding sequence of *TA* 27 was not in frame with beta-galactosidase in the pBluescript vector, the coding sequence was sub-cloned into the expression vector pET 11a (Novagen), with translation starting at the first ATG codon in the clone, which gave a reasonable alignment of the open reading frame with that of other GSTs involved in herbicide metabolism, notably the *Zm*GSTIV sequence.

(d) Activity of recombinant GSTs of the invention

To confirm that the *WIC* clones and *TA 27* encoded functional GSTs the corresponding enzymes were expressed as recombinant enzymes in *E. coli*. The full coding sequence of *TA 27* was expressed in the pET vector, while the WIC clones were expressed as fusions with part of the beta-galactosidase enzyme using the pBluescript vector. The levels of recombinant protein expressed varied between the differing clones. Appreciable amounts of recombinant protein were observed in the *TA 27* pET clones and in clones *WIC 1, WIC 2, WIC 4* and *WIC 5*. Western blotting of these total bacterial extracts with the antiserum raised to *Zm*GSTI-II showed that the fusion proteins were selectively recognised by the antiserum. In contrast, use of the antiserum demonstrated much lower levels of expression of immunoreactive fusion proteins in

clones *WIC 3, WIC 7, WIC 8* and *WIC 10.*

To assay the recombinant fusion proteins for GST activity , the *E. coli* were grown in the presence of IPTG and then pelleted by centrifugation. The bacteria were then lysed by sonication and the protein precipitated using 80% ammonium sulphate. After resuspension and desalting, GSTs were purified by affinity chromatography. The WIC 3 fusion protein was purified using sulphobromophthalein-*S*-glutathione affinity chromatography (Mozer *et al.* Biochem. 22, 1983, 1068-1072) while the other WIC fusion proteins were purified using glutathione-agarose (Mannervik and Guthenberg. Methods Enzymol. 77, 1981, 231-235).The purified enzymes were then assayed for GST activities toward herbicides (Table 3) and GST activities toward non-herbicide substrates and glutathione peroxidase activities toward organic hydroperoxides (Table 4).

Table 4

Other GST activities and glutathione peroxidase activities.

Activities expressed as nkat.mg^{-1} ± standard error. Peroxidase activities expressed as absorbance change at 340 nm.mg^{-1} ± standard error (n=3). N.D = not detected, - not performed.

	CUMENE HYDROPEROXIDE	BENZYL ISOTHIOCYANATE	CROTONALDEHYDE	ETHACRYNIC ACID
WIC 1	18.6 ± 0.5	18.0 ± 3.75	7.1 ± 0.7	N.D.
WIC 2	28.2 ± 1.7	33.3 ± 4.5	5.5 ± 1.3	N.D.
WIC 3	1.4 ± 0.3	9.0 ± 0.5	6.3 ± 0.9	N.D.
WIC 4	6.2 ± 0.3	4.2 ± 0.4	5.5 ± 0.6	1.4 ± 0.3
WIC 5	1.3 ± 0.2	9.4 ± 2.0	4.5 ± 0.5	N.D.
TaGST1	0.7 ± 0.1	11.8 ± 0	3.7 ± 0.3	N.D.
TA 27	3.6 ± 0.4	-	-	-
ICR	N.D	-	-	-
ICC/V/P	0.84 ± 0.04	-	-	-

Example 3:Cloning of safener-inducible type III GSTs from wheat.

A polyclonal antiserum was raised in a rabbit to a mixture of TaGST1-2 and TaGST1-3. When tested against crude extracts from safener-treated wheat shoots the antiserum recognised both the 25 kDa TaGST1 subunit and the 26 kDa safener-inducible TaGST2 and TaGST3 subunits. The antiserum was then used in conjunction with the antiserum raised to TaGST1-1 to immuno screen the cDNA library prepared from fenchlorazole ethyl treated wheat shoots as described in example 1. Duplicate lifts were taken from the plated out library and the first blot screened with the antiserum raised against *Ta*GST1-2 and *Ta*GST1-3. The second blot was screened with the antiserum raised to *Ta*GST1-1. Five plaques were identified from the first blot which were absent from the second blot corresponding to cDNAs encoding *Ta*GST2 or *Ta*GST3 like polypeptides and theses clones were purified and the respective plasmids sequenced. One of the clones, termed ICJ had an identical nucleotide sequence to *GST Ts1*, a safener-inducible GST identified in *Triticum tauschii* (Riechers et al., 1997 Plant Physiol. 114, 1568). Another clone, ICR, though showing some similarity to ICJ had a novel coding DNA coding sequence (SEQ ID No. 15) and predicted amino acid sequence (SEQ ID No. 16). The other three clones ICC. ICP and ICV had identical DNA sequences (SEQ ID No. 17) and predicted amino acid sequence (SEQ ID No. 18). GST ICJ was sub-cloned into the pET 11a vector after using PCR to introduce a Nde 1 restriction site into the translation start site, using the primer 5' AGG TAG TTA CAT ATG GCC GGA GGA 3' (SEQ ID No. 19) in the amplification. following sub-cloning, the sequence of GST ICJ was re-checked to ensure no PCR induced errors had been introduced. The recombinant GST ICJ was then expressed in *E.coli* and purified by *S*-hexylglutathione affinity chromatography. The purified GST ICJ was assayed for activities as a GST (Table 3) and as a glutathione peroxidase (Table 4). The clone GST ICV was expressed in a variety of vectors, but in all cases the recombinant proteins proved impossible to purify using a variety of affinity columns (*S*-hexylglutathione-agarose , *S*-bromosulphophthalein glutathione agarose). GST ICV was finally expressed as the respective beta-galactosidase fusion protein using the Bluescript plasmid and assayed for GST activity (Table 3) and glutathione peroxidase activity

(Table 4) in crude bacterial lysates. The specific activity of GST ICV toward these substrates was then calculated by I) subtracting the low levels of GST and GPOX activity present due the endogenous activities in the *E.coli* and ii) determining the proportion of protein in the lysate present as recombinant GST ICV from SDS-PAGE analysis, by densitometry analysis of polypeptides stained with Coomassie blue .

EXAMPLE 4: A microtitre plate - based screen to identify herbicidal molecules which are metabolised by GSTs of the invention and may selectively control weeds in a crop of wheat or other species such as maize, soybean or rice

(a) Degradation of candidate herbicides by wheat GSTs and relationship to crop and weed selectivity

Herbicidal molecules which are degraded by recombinant wheat GSTs may be predicted to be tolerated by plants of wheat or other crop species. These herbicides may be less rapidly degraded in weeds such as black-grass (*Alopecurus myosuroides*) which are desirable to control in a crop of wheat or other species. Herbicides found in a laboratory based screen to be metabolised by these GSTs are therefore likely to possess useful abilities to selectively control troublesome weeds in a crop of wheat, or other species such as maize, soybean, rice, cotton, barley, oat, rye, sorhum, triticale, potato, sugarcane or sugarbeet.

(b) A 96 well plate - based assay procedure for identifying novel herbicides degraded by recombinant wheat GSTs

Novel herbicides arising from a chemical synthesis programme oriented to optimisation for selective herbicidal activity and potency may be screened for ability to be degraded by a panel of recombinant GSTs using a 96 well microplate assay format and subsequent reaction analysis by automated High Pressure Liquid Chromatography (HPLC). This allows for example, the screening of a set of eleven novel herbicides and one positive control compound such as CDNB, against a panel of seven recombinant

GSTs. An eighth file of wells contains test compounds but lacks GSTs; these wells serve to identify non-enzymic reaction of the test compounds with reduced glutathione. Alternatively, the array can be configured to screen more test compounds against a more limited number of GSTs. For example, fifteen compounds can be screened against five GSTs or forty seven compounds may be screened with a single mixture of GSTs. In all cases, provision is made for a positive control and to test for non-enzymic reaction with reduced glutathione.

Enzyme assays are carried out in a total reaction volume of 100 microlitres. Each reaction mixture contains 100 micromolar Tris.HCl buffer, pH 7.8, 500 micromolar reduced glutathione and where appropriate, 500 micromolar test compound or a reference substrate such as CDNB; and 14 micrograms of GST protein. The microplate is incubated at 30°C on a variable speed agitator for 30 minutes and reactions are then stopped by the addition of 15 microlitres of 23% perchloric acid solution. The microplate is then centrifuged at 2000 g for 15 minutes.

(c) Reaction analysis by automated High Pressure Liquid Chromatography.

The separation and analysis of glutathione conjugates of test herbicides may be carried out using High Pressure Liquid Chromatography (HPLC), for example a Gilson HPLC in tandem with corresponding software, for example Gilson Version 7.12 and fitted with an appropriate column, for example a 5 cm Spherisorb ODS2 column. Typically, separation may be carried out using a two phase solvent system as follows: Phase A: water containing 0.1% trifluoroacetic acid and 5.5% acetonitrile; Phase B: 100% acetonitrile; flow rate 1.5 ml per minute; injection volume 20 microlitres.

The elution gradient may be typically as follows: 10% phase B for one minute, followed by a linear gradient to reach 60% phase B after 8.5 minutes. The gradient is further increased to reach 100% phase B at 9 minutes; phase B is continued at 100% until 11.5 minutes and is then reduced in a linear gradient to 10% at 13.5 minutes. A further 1.5 minutes at 10% phase B is required to re-equilibrate the column.

Absorbance signals are detected at 264 nanometres using a suitable UV detector, and detect the glutathione conjugate of CDNB, having a retention time of 2.4 minutes, resolving this from unreacted CDNB having a retention time of 4.75 minutes. Such conditions also allow for the resolution and detection of the glutathione conjugates arising from the metabolism of other reference herbicides such as metolachlor, fenoxaprop, fenoxaprop-ethyl and fluorodifen and also of a variety of novel herbicidal compounds identified in the assay.